Pflügers Arch – Eur J Physiol (2000) 439:416–422
Digital Object Identifier (DOI) 10.1007/s004249900180

ORIGINAL ARTICLE

W.M. Aartsen · M.M.A.L. Pelsers · W.T. Hermens
J.F.C. Glatz · M.J.A.P. Daemen · J.F.M. Smits

Heart fatty acid binding protein and cardiac troponin T plasma concentrations as markers for myocardial infarction after coronary artery ligation in mice

Received: 15 July 1999 / Accepted: 27 September 1999 / Published online: 30 October 1999

Abstract Ligation of the main left coronary artery in mice serves as a model for myocardial infarction (MI). We tested whether plasma concentrations of heart-type fatty acid-binding protein (H-FABP) and/or cardiac troponin T (cTnT) discriminate between infarcted and sham-operated mice and allow estimation of infarct size. Mice were subjected to coronary artery ligation or sham surgery and release curves of H-FABP and cTnT were determined. At 4 h after surgery the mean (±SD) H-FABP plasma concentration was 461±134 µg/l (n=10) in MI and 185±51 µg/l (n=6; P<0.001) in sham-operated mice. By 24 h after surgery H-FABP levels had returned to normal in both groups. cTnT plasma concentrations increased up to 48 h after MI to 13.5±6.2 µg/l (n=6; P<0.001) compared with 0.031±0.063 µg/l (n=7) in sham-operated mice. Linear regression analysis revealed a significant correlation between plasma H-FABP at 4 h and infarct size assessed 7 days after surgery. Plasma cTnT did not correlate significantly with infarct size. In conclusion, plasma cTnT concentration at 48 h after infarction can be used to distinguish MI from sham mice, whereas H-FABP concentration at 4 h can be used for stratification of animals according to infarct size.

Key words Diagnostic Techniques · Myocardial ischaemia · Mice

W.M. Aartsen (✉) · J.F.M. Smits
Department of Pharmacology and Toxicology,
Cardiovascular Research Institute Maastricht (CARIM),
Universiteit Maastricht, PO Box 616, NL-6200 MD Maastricht,
The Netherlands
e-mail: wendy.aartsen@farmaco.unimaas.nl
Tel.: +31-433881342, Fax: +31-433884149

M.M.A.L. Pelsers · J.F.C. Glatz
Department of Physiology, CARIM, Universiteit Maastricht,
Maastricht, The Netherlands

W.T. Hermens
CARIM, Universiteit Maastricht, Maastricht, The Netherlands

M.J.A.P. Daemen
Department Pathology, CARIM, Universiteit Maastricht,
Maastricht, The Netherlands

Introduction

Permanent occlusion of the main left coronary artery in mice results in antero-apical myocardial infarct (MI) with features such as ventricular dilation, wall thinning and septum hypertrophy which are highly comparable to those in rats [15]. In most experiments animals are stratified into groups with different interventions. To distinguish between sham-operated and infarcted animals and to construct groups with comparable infarct sizes at the start of each study, a method is needed not only to confirm the presence of MI, but also to quantify infarct size. Ideally, both parameters should be available at an early time. In patients, several criteria have been defined by the World Health Organisation [31] for diagnosing acute MI including electrocardiographic changes and increases in plasma concentrations of cardiac proteins.

Clinically, the enzymes aspartate aminotransferase (ASAT), creatine kinase (CK) and lactate dehydrogenase (LDH) are used as plasma markers for the detection of cardiac injury. Although isoforms of CK and LDH, like CK-MB, LDH_1 and LDH_2, are highly expressed in myocardium, these markers are not cardiac specific. Increased levels of these enzymes are also observed in clinical settings without myocardial injury (e.g. trauma and various inflammatory myopathies) [1, 17]. Recently, new plasma marker proteins such as myoglobin, heart-type fatty acid-binding protein (H-FABP) and cardiac troponin T and troponin I (cTnT/cTnI) have been introduced, allowing a faster and better diagnosis [10, 19, 22, 28, 30]. H-FABP and myoglobin are detectable in the circulation 4 h after the onset of anginal complaint, which makes them useful as early markers [1, 26]. Although cTnT and cTnI are released only slowly into the circulation these markers are more specific for myocardial injury than CK and LDH [2, 6, 9, 11, 18]. Thus, both H-FABP (as an early marker) and cTnT/cTnI (as a specific marker) are potential candidates for biochemical determination of myocardial damage after experimental coronary artery ligation in mice.

FABPs are present in the cytoplasm of fatty acid-utilising cells in liver, skeletal muscle and heart [21]. They

constitute a family of small proteins (14–16 kDa) involved in the cellular transport and metabolism of fatty acids [3]. Heart-type FABP is abundant in the cytoplasm of myocardial cells [8]. Following cell damage by acute myocardial ischaemia, H-FABP is released rapidly into the circulation, from which it is cleared unchanged by the kidneys. This makes it possible to determine H-FABP levels both in plasma and urine [13]. In humans, elevated plasma and urine levels are found within 3 h after the onset of anginal complaint [7, 25]. Moreover, in these patients a significant correlation is found between the amount of released H-FABP and the infarct size [7]. Similar results have been demonstrated after experimental MI in rats: significantly more H-FABP is found in 24-h urine from rats with MI compared with sham-operated rats [27].

Troponins are part of the contractile unit in muscle cells. The troponin complex contains three different troponin polypeptides: troponins T (37 kDa), I (24 kDa) and C (18 kDa) [23]. Troponins T and I can be used as cardiac-specific markers, since heart muscle expresses a unique troponin T and I isoform (cTnT/cTnI) [23, 29]. cTnT and cTnI have been evaluated for the diagnosis of myocardial injury. Increased cTnT and cTnI levels are found after MI [16] and in myocarditis [24]. More recently O'Brien et al [20] have demonstrated that cTnT is a useful marker for confirming doxorubicin-induced myocardial damage in mice.

The present study investigated the possibility of estimating the infarct size by analysis of a single blood sample. To determine a suitable time and to perform linear regression analysis between the plasma H-FABP or cTnT concentrations and the percentage of left ventricle infarcted, a short-term and a long-term study were designed. In the short-term study Swiss mice were subjected to experimental coronary artery ligation followed by blood sampling for the measurement of H-FABP and cTnT plasma concentrations 1, 2, 4, and 6 h after surgery. In the long-term study blood was sampled 4, 24 and 48 h after surgery. Hearts were fixed 7 days after surgery and infarct sizes determined histologically. Linear regression analysis was performed with the biochemically determined marker levels. The data show that cTnT is a highly sensitive and specific marker for the detection of myocardial injury in mice. However we found no correlation between cTnT plasma concentrations (48 h) and infarct size. H-FABP plasma concentrations at 4 h correlated well with the infarct size determined histologically 7 days after surgery.

Materials and methods

Animals

Adult (10-week-old), male, Swiss mice (Iffa Credo, The Netherlands) weighing 35–45 g at the time of surgery were used. Animals were housed in groups of between four and eight and had free access to standard food (SRMA-1210; Hope Farms, Woerden, The Netherlands) and tap water. All experiments were conducted according to institutional guidelines.

Experimental design

Two types of experiments were conducted. The short-term study was designed to estimate the time-release curve of H-FABP and cTnT. For this, the aorta was cannulated and blood sampled 1, 2, 4 and 6 h after surgery. The long-term study was designed to analyse the correlation between a single blood sample and the eventual infarct size 7 days after surgery. In the long-term study animals were subjected to surgery followed by blood sampling via puncture of the orbital plexus at 4, 24 and 48 h after surgery. The hearts were fixed 7 days after surgery and infarct sizes determined.

Coronary artery ligation

The method for establishing MI in mice has been described elsewhere [15]. Briefly, the animal was anaesthetised with pentobarbitone sodium (110 mg/kg i.p.) and the trachea intubated and connected to a pressure-cycled ventilator (1.5–2 ml, 70/min). After opening the skin, the left 4th intercostal space and the pericardial sac, a ligature (6-0 prolene) was tied around the main left coronary artery. Then chest and skin were closed with 5-0 silk sutures under application of gentle pressure on both side of the thorax to expel air, after which the animal was allowed to recover. Sham surgery was performed identically except for the ligature around the left coronary artery.

Blood sampling

For the short-term experiments, a catheter (PE 25 tubing) was implanted in the course of the surgery session in the abdominal aorta via the femoral artery. The saline-filled catheter was exteriorised at the neck and closed with a metal plug. Blood samples (200 µl) were collected in heparinised tubes 1, 2, 4 and 6 h after ligation; samples were replaced by equal volumes of saline. In the, long-term study, blood was obtained by puncture of the orbital plexus under ether anaesthesia 4, 24 and 48 h after surgery. After centrifugation of the blood for 10 minutes at 10000 *g*, plasma was stored at –20 °C until assayed for H-FABP and cTnT.

Immunoassay for H-FABP

A sandwich-type, enzyme-linked immunosorbent assay (ELISA) for rodent H-FABP was provided by Hycult Biotechnology, Uden, The Netherlands. In short, 50 µl of the second monoclonal antibody against H-FABP (conjugated with horseradish peroxidase, HRP) and 50 µl of either the diluted plasma or the H-FABP standards (0–25 µg/l) were added first to the pre-coated plate. After incubation for 3 h at room temperature, the wells were washed 4 times with 200 µl buffer followed by the addition of 100 µl substrate solution. After 15 min the reaction was stopped by adding 100 µl stop solution and the absorbance at 450 nm measured using a Titertek Multiscan MKII microplate reader. The detection limit of the assay was 0.1 µg/l H-FABP and the inter- and intraassay analytical imprecision were below 10%.

Immunoassay for cTnT

Plasma cTnT concentrations were determined using the second-generation cTnT enzyme-linked immunoassay (Enzymun-test Troponin-T; Boehringer Mannheim, Indianapolis, Ind., USA) [10]. Undiluted plasma (50 µl) or standards (0–18.8 µg/l) were added to the wells of polyvinyl microtitre plates pre-coated with streptavidin. Then 200 µl phosphate buffer (40 mM; pH 7.0) containing both biotinylated anti-troponin-T antibodies M7 (1.5 mg/l) and HRP-labelled anti-troponin-T antibody M11.7 (>100 U/L) were added, followed by an incubation for 1 h at room temperature. According to the manufacturer's instructions, the wells were washed 3 times with sodium perborate (3.2 mM), 2,2-azino-bis 3-ethylbenzothiazoline-6-sulphonate substrate (1.9 mM in 0.1 mM phosphate buffer) was

added and, after 15 min incubation, the absorbance measured at 405 nm using a Titertek Multiscan MKII microplate reader.

Measurement of infarct size

One week after surgery all animals were killed by an overdose of pentobarbitone sodium. Hearts were weighed and fixed in 10% phosphate buffered formalin for 24 h. After fixation the hearts were cut longitudinally through the left and right ventricles, dehydrated and paraffin embedded. Sections of 4 µm were cut from both heart halves and stained with the AZAN technique. These sections were analysed by a computerised morphometry system (Quantimet 570, Leica, The Netherlands). MI size was measured as percentage of the left ventricle circumference, taking the average of both heart halves [15].

Statistics

Data are presented as mean±SD. Sensitivity and specificity are calculated from the long-term study. Sensitivity was defined as the percentage of correctly diagnosed infarctions. Specificity was defined as the percentage of correctly diagnosed non-infarctions. The positive predictive value is defined as the percentage of correct positive tests and the negative predictive value as the percentage of correct negative tests. Statistical analysis was performed using unpaired Student's *t*-tests and linear regression analysis. $P<0.05$ was regarded as statistically significant.

Results

General

A total of 53 animals were subjected to surgery. For the short-term study 9 animals were sham operated, of which 8 (89%) survived; 15 animals were subjected to coronary artery ligation of which 11 (73%) survived. These mice were cannulated for blood sampling 1, 2, 4, and 6 h after surgery. Animals were under anaesthesia until approximately 3 h after surgery. Three mice that had not regained consciousness 4 h after surgery (two sham-operated and one MI) were excluded and died within 24 h, leaving six sham-operated and ten infarcted mice included in the short-term study. For the long-term study 9 of the 10 sham-operated animals survived (90%) and 10 of the 19 animals subjected to coronary artery ligation survived (53%). One infarcted mouse failed to regain consciousness after 4 h and was excluded. Thus, for the later times nine sham-operated animals and nine animals with MI were re-anaesthetised with ether to puncture the orbital plexus 4, 24 and 48 h after surgery.

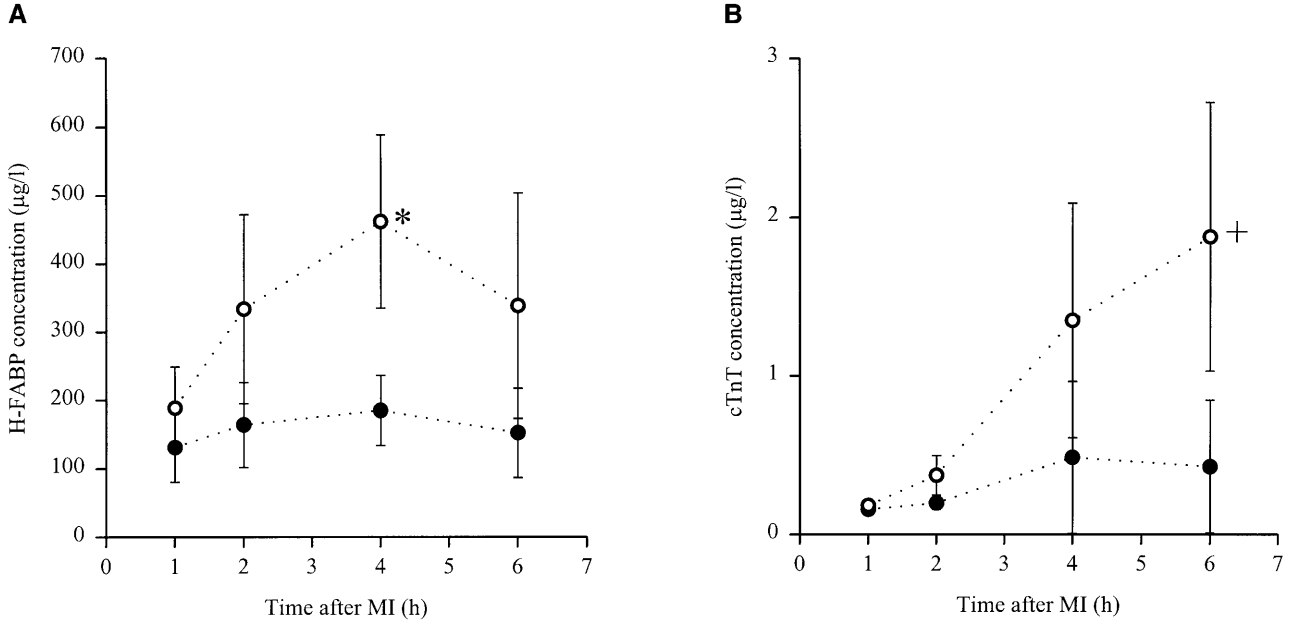
H-FABP plasma concentrations

The time-course of plasma H-FABP after MI is shown in Fig. 1. Within 2 h after surgery H-FABP plasma concentration was above control (33±28 µg/l) and peak levels of H-FABP were measured at 4 h. At that time significantly higher concentrations were measured in animals with MI than in sham-operated animals (461±134 µg/l vs. 185±51 µg/l, $P<0.05$).

In the long-term study (Fig. 2), plasma H-FABP was raised in both sham and MI animals. Again a significant difference was found between H-FABP plasma concentrations of sham-operated and MI animals at 4 h. H-FABP plasma concentrations of both groups returned to baseline level at 24 h after surgery.

In hearts from animals included in the second experiment, infarcted areas were measured as percentages of the left ventricle. The infarcts occupied 29.9–59.5% of the left circumference and the infarct size correlated significantly ($P=0.02$) with plasma H-FABP at 4 h (Fig. 3).

Fig. 1 Short-term study: plasma heart fatty acid-binding protein (*H-FABP*, *left panel*) and cardiac troponin T (*cTnT*, *right panel*) concentrations measured 1, 2, 4 and 6 h after coronary artery ligation causing myocardial infarction (*MI*) in mice. Means±SD; sham-operated (*solid circles*, $n=6$); MI (*open circles*, $n=10$) mice, $^{+}P<0.05$, $^{*}P<0.001$



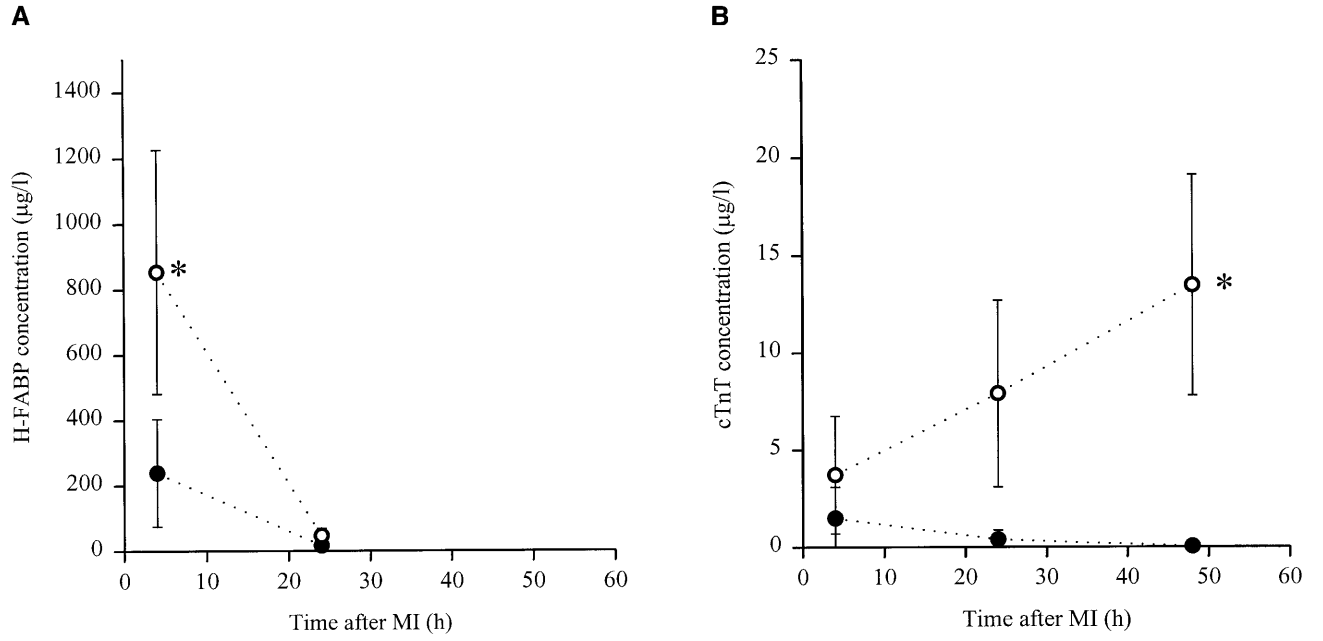


Fig. 2 Long-term study: plasma H-FABP (*left panel*) and cTnT (*right panel*) concentrations 4, 24 and 48 h after surgery. Means±SD, sham (*solid circles*, *n*=9) and MI (*open circles*, *n*=9), **P*<0.001

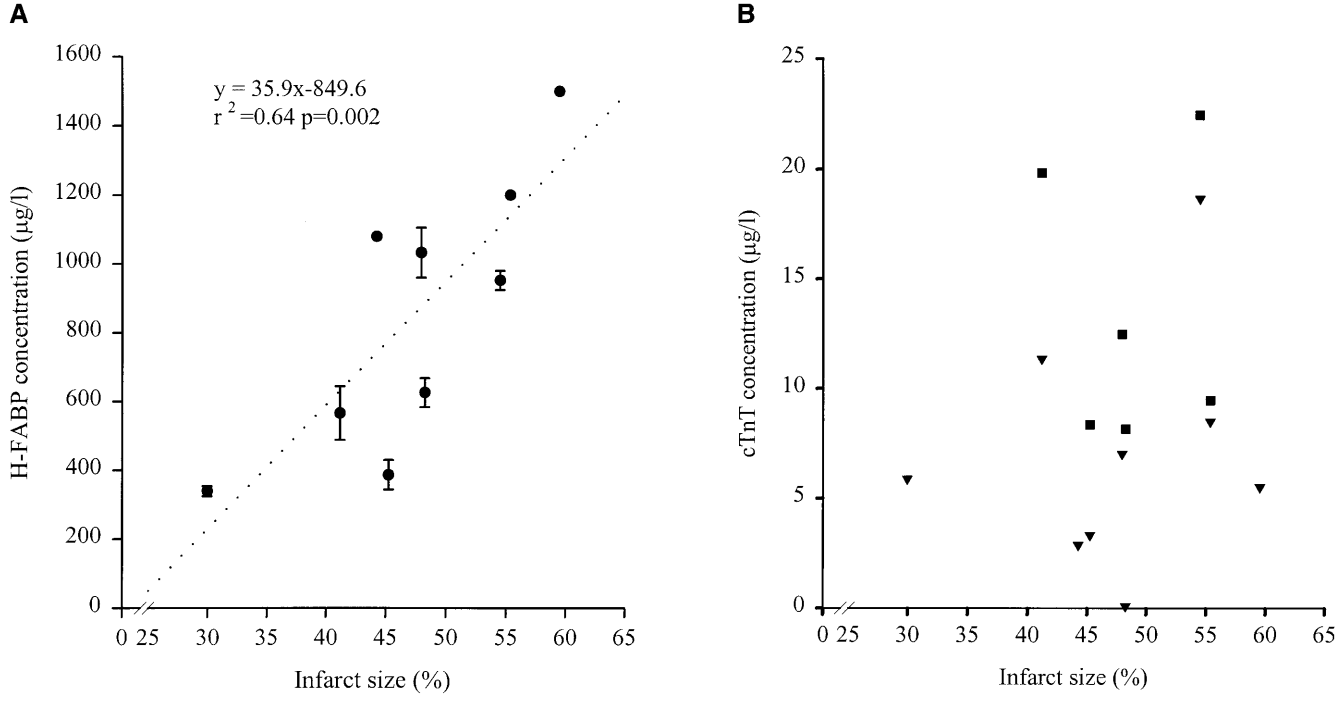


Fig. 3 Relationship between infarct size and plasma concentration of H-FABP (*left panel*) and cTnT (*right panel*). *Left panel*: linear regression analysis of plasma H-FABP 4 h after MI, derived from two separate ELISA measurements (means±SD; *solid circles*) and the percentage of the left ventricle infarcted. *Right panel*: lack of correlation between plasma cTnT 24 (*solid triangles*) and 48 (*solid squares*) h after MI and the percentage of the left ventricle infarcted, measured 7 days after MI

Using H-FABP as a marker for MI in mice, a sensitivity of 78% and a specificity of 89% was reached. The positive predictive value of this test was 88% and the negative predictive value 80%.

cTnT plasma concentrations

cTnT plasma concentrations were increased in both groups within 6 h after surgery and at 6 h a significant

difference was found between mice with MI (n=10) and sham operated animals (P<0.05, n=4, Fig. 1).

In the long-term study, plasma cTnT in sham operated mice returned to baseline level (0.031±0.063 ng/ml, n=9) after 24 h while mean cTnT plasma concentration of infarcted animals continued to increase up to 48 h after MI (13.46±6.21 ng/ml, n=9; Fig. 2). Plasma cTnT did not correlate with the infarct size at either time. Nevertheless, cTnT measured 48 h after MI in mice is a highly sensitive (100%) and specific (100%) marker for the presence of myocardial injury. As test for myocardial injury, cTnT concentration at 48 h after MI had both positive and negative predictive values of 100%.

Discussion

Previous results have indicated that ligation of the main left coronary artery in mice results in remodelling of the heart comparable to that observed in rats [15]. The early recognition of successfully infarcted animals and the non-invasive estimation of infarct size is a major problem in studies on the effects of interventions on cardiac function and remodelling. We thus sought a method allowing assessment of cardiac necrosis in mice early after coronary artery ligation. The results indicate that cTnT plasma concentration 48 h after surgery is a highly specific and sensitive indicator for the presence of cardiac necrosis. In addition, plasma H-FABP concentration at 4 h, although less sensitive and specific, correlates with infarct size.

In humans, elevated plasma H-FABP levels are found within 3 h after the onset of anginal complaint. Moreover, in these patients a significant correlation is found between the amount of released H-FABP and the infarct size [7]. Similar results were obtained in the present study. At 4 h after surgery, H-FABP plasma concentrations were significantly increased in mice with histologically verified infarcts compared with sham-operated animals. Within 24 h H-FABP plasma levels had returned to baseline levels. The increased H-FABP concentrations in sham-operated animals are most likely explained by H-FABP expression in skeletal muscle [26]. Since H-FABP concentrations measured 4 h after surgery correlate with the extent of infarction established histologically 7 days after surgery, it is possible to estimate infarct size within a few hours after surgery.

In patients suffering myocardial infarction, cTnT and cTnI serum levels are increased within 6 h after the onset of chest pain and remain elevated for at least 4 days [1, 5, 14]. In the present study we measured plasma cTnT concentrations until 48 h after surgery and mice with MI exhibited steadily increasing cTnT plasma levels up to 48 h. The slightly elevated levels in sham-operated animals may have been caused by cross-reactivity of the antibody with skeletal muscle troponin T. Due to the prolonged elevation of the plasma levels, cTnT is a late (48 h after MI) but very sensitive (100%) and specific (100%) plasma marker. Despite this, no correlation was found between the infarct size and cTnT concentrations measured 4, 24 or 48 h after surgery.

Higher concentrations for both H-FABP and cTnT were consistently found in the long-term study than in the short-term study. The reason for this discrepancy is not clear, but could derive from the different methods used to collect the blood samples. Due to these differences the results should be interpreted separately. Only the H-FABP and cTnT concentrations obtained from the long-term study, in which blood was obtained by puncture of the orbital plexus were related to infarct size.

There are several possible explanations for the apparent lack of correlation between infarct size and the plasma concentration of cTnT. The applicability of measurements of cardiac proteins in plasma as indicators for cardiac injury depends upon several assumptions. Firstly, the protein studied must be of (preferential) cardiomyocyte origin. Secondly, it must be contained within the cell under normal conditions and leak out upon cell death. This is the case for both H-FABP and cTnT. Independent of the rate of leakage, the plasma concentration-time integral of a protein that originates exclusively from cardiomyocytes will depend on the number of myocytes that expel their cytoplasm and the concentration of the particular protein therein. Assuming that the latter is constant, the integral should reflect the extent of cell death. However, the small blood volume in mice prohibits the frequent sampling that would be required to determine the integral. Alternatively, if the protein studied is excreted via the kidneys, quantitative collection of urine over a prolonged period would allow measurement of the total amount of protein expelled. Again, the small amount of concentrated urine secreted by mice makes this approach impractical. Measurement of a plasma concentration at a single time is practical, but the predictive value of such measurements depends upon the kinetics of release into, and excretion from plasma. The former depends upon diffusion, and thus the rate of leakage is negatively correlated with the size of the molecule. Since cTnT is about 2.5 times larger than H-FABP it may be expected to enter the circulation more slowly than the latter. Leakage of cTnT is further slowed by the fact that only approximately 5% is present in a free form; most cTnT is complexed within the cardiomyocytes [4, 12]. As demonstrated in this study H-FABP plasma concentrations are more rapidly normalised than those of cTnT, suggesting that the clearance rate of H-FABP is much higher. Katus et al, on the other hand, have suggested that the serum half-life of cTnT in humans is only 2 h [12, 30]. Together with the fact that mice with myocardial infarction had increasing cTnT plasma concentrations until 48 h after surgery and sham-operated animals returned to baseline levels within 24 h suggests ongoing release of cTnT rather than

very slow clearance kinetics. Hence, a single determination of plasma cTnT should be less sensitive to differences in the extent of the infarct than the rapidly released and cleared H-FABP. Alternatively, it has been suggested that cTnT is not only released into the circulation in its free form, but may circulate as a complex with cTnI and/or cTnC [5]. To detect all cTnT released from the heart after MI, the antibodies employed should be capable of recognizing both free and complexed cTnT. Whether this is so for the antibodies used in this study is unknown.

The present study describes a method for estimating the experimentally induced infarct size in mice with a single blood sample. Before stratification of the animals into different groups it is often useful to know if the induced coronary artery ligation has been successful. Both H-FABP and cTnT can be used as plasma markers of myocardial damage in mice after experimentally induced MI. H-FABP plasma concentration 4 h after surgery correlates with the infarct size measured histologically 7 days after MI. cTnT plasma concentration 48 h after surgery does not relate to infarct size, but may serve as a very reliable qualitative marker.

Acknowledgements The authors wish to thank J.J.M. Debets, P.J.A. Leenders, N.J.J.E. Bitsch and M.L.L. Mullers-Boumans for their technical assistance. We wish to thank Hycult Biotechnology, Uden, for providing reagents. This study was supported in part by the Ministry of Economic Affairs; grant BTS 97.188.

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